

EXHIBIT A
J. Smith, et al.

PTEN Mutation, EGFR Amplification, and Outcome in Patients With Anaplastic Astrocytoma and Glioblastoma Multiforme

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Background: Survival of patients with anaplastic astrocytoma is highly variable. Prognostic markers would thus be useful to identify clinical subsets of such patients. Because specific genetic alterations have been associated with glioblastoma, we investigated whether similar genetic alterations could be detected in patients with anaplastic astrocytoma and used to identify those with particularly aggressive disease. **Methods:** Tissue specimens were collected from 174 patients enrolled in Mayo Clinic Cancer Center and North Central Cancer Treatment Group clinical trials for newly diagnosed gliomas, including 63 with anaplastic astrocytoma and 111 with glioblastoma multiforme. Alterations of the EGFR, PTEN, and p53 genes and of chromosomes 7 and 10 were examined by fluorescence *in situ* hybridization, semi-quantitative polymerase chain reaction, and DNA sequencing. All statistical tests were two-sided. **Results:** Mutation of PTEN, amplification of EGFR, and loss of the q arm of chromosome 10 were statistically significantly less common in anaplastic astrocytoma than in glioblastoma multiforme ($P = .033$, $P = .001$, and $P < .001$, respectively), and mutation of p53 was statistically significantly more common ($P < .001$). Univariate survival analyses of patients with anaplastic astrocytoma identified PTEN ($P = .002$) and p53 ($P = .012$) mutations as statistically significantly associated with reduced and prolonged survival, respectively. Multivariate Cox analysis of patients with anaplastic astrocytoma showed that PTEN mutation remained a powerful prognostic factor after adjusting for patient age, on-study performance score, and extent of tumor resection (hazard ratio = 4.34; 95% confidence interval = 1.82 to 10.34). Multivariate classification and regression-tree analysis of all 174 patients identified EGFR amplification as an independent predictor of prolonged survival in patients with glioblastoma multiforme who were older than 60 years of age. **Conclusion:** PTEN mutation and EGFR amplification are important prognostic factors in patients with anaplastic astrocytoma and in older patients with glioblastoma multiforme, respectively. [J Natl Cancer Inst 2001;93:1246-56]

Diffuse astrocytomas are a heterogeneous collection of glial cell neoplasms that exhibit a remarkable range of morphologic features and clinical behavior (1). The current World Health Organization (WHO) guidelines distinguish three malignancy grades (grades 2-4) on the basis of histologic features that predict patient survival (2). Whereas glioblastoma multiforme (WHO grade 4 astrocytoma) is associated with a uniformly poor outcome, survival varies considerably among patients with low-grade astrocytoma (WHO grade 2) and anaplastic astrocytoma (WHO grade 3) (3). Our ability to stratify these patients contin-

ues to be limited primarily to histologic grading and clinical parameters, such as age and performance score (1,4-7). However, these parameters do not fully account for the observed variation in survival (3,8), and additional indicators are needed to more accurately determine prognosis and to identify novel therapeutic approaches that minimize patient morbidity and mortality. In this regard, the further stratification of anaplastic astrocytomas is extremely important, because survival for patients with these tumors ranges from several years (often observed for patients with low-grade astrocytomas) to just a few months (observed with most patients with glioblastoma multiforme) (3).

Multiple genetic alterations have been identified in astrocytic gliomas and linked together as a sequence of events whose occurrence parallels the malignant progression of these tumors (1,9). The association of genetic alterations with astrocytic glioma behavior has stimulated multiple investigations into the prognostic relevance of genetic markers (1). These studies have primarily focused on the more common glioblastoma multiforme and have included examination of the PTEN and p53 tumor suppressor genes, amplification of the EGFR oncogene, loss of the q arm of chromosome 10, and gain of chromosome 7.

In this study, we have examined tumors from a large cohort of uniformly treated patients with anaplastic astrocytoma for specific alterations in the p53, EGFR, and PTEN genes and of chromosomes 7 and 10 to determine whether a subset of these patients with particularly aggressive disease can be identified. For cross-grade comparison, we also analyzed a large collection of patients with glioblastoma multiforme treated on similar prospective clinical trials for the same alterations.

SUBJECTS AND METHODS

Tumors and Patients

Tissues from 174 patients were studied. The anaplastic astrocytoma subgroup included all 63 patients with biopsy-proven WHO grade 3 astrocytoma and sufficient tissue for the marker studies to be performed. These patients were enrolled in one of three consecutive North Central Cancer Treatment Group (NCCTG) phase III trials for newly diagnosed high-grade gliomas, protocols 79-72-51, 85-72-51, and 88-72-52 (10-12). The glioblastoma multiforme con-

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trial group included all 111 Mayo patients with newly diagnosed, biopsy-proven, WHO grade 4 astrocytoma and sufficient tissue for marker ascertainment. These patients were enrolled in the same three NCCTG trials, six concurrent Mayo Clinic Cancer Center (MCCC) high-grade glioma trials, or a four-arm NCCTG-led intergroup trial for newly diagnosed glioblastoma multiforme. Clinical information and survival information were available for both groups.

All tissue specimens were obtained at initial diagnosis by resection or by biopsy before initial resection and were classified morphologically and graded according to the current WHO system (2). All marker studies were performed after receiving approval from the institutional review boards from each of the participating institutions. All patients gave written informed consent to participate in the parent NCCTG clinical trials.

Patient entry in the parent NCCTG clinical trials required pathologic evaluation of all relevant surgical specimens by a single neuropathologist (B. W. Scheithauer). Because the biologic studies consumed a large portion of the paraffin blocks, the anaplastic astrocytomas were rereviewed by Dr. Scheithauer and another neuropathologist (Dr. Arie Perry, Washington University, St. Louis, MO). This rereview found evidence of tumor heterogeneity and sampling bias but did not change the conclusion of the study (see the "Discussion" section).

For each NCCTG trial, patients were stratified before randomization by age, extent of surgery, tumor grade, and performance score. All patients received cranial radiation therapy and chemotherapy. No differences in survival were identified between the randomized treatment arms in any of the trials, although a modest overall increase in survival from the first to the third NCCTG trial was noted (10–12). The six concurrent MCCC trials were one-arm pilot studies designed to gain preliminary information about the efficacy and toxicity of new therapies. Typical treatment included surgical resection followed by radiation therapy and adjuvant chemotherapy with either a single chemotherapeutic agent, such as carmustine, or a combination of agents, such as cisplatin, etoposide, and carmustine. Several of these regimens were incorporated into subsequent NCCTG phase III trials.

The on-study performance score was based on a scale of 0–4, as described previously (13,14). In general, 0 = fully active, 1 = mildly restricted physical activity, 2 = ambulatory more than 50% of the day, 3 = confined to bed or chair more than 50% of the day, and 4 = totally confined to bed or chair.

Genetic Analysis

Dual-probe fluorescence *in situ* hybridization (FISH) analyses were performed on paraffin-embedded sections as described previously (15), with locus-specific probes for EGFR and PTEN paired with centromere probes for chromosomes 7 (CEP7) and 10 (CEP10), respectively (Vysis, Downers Grove, IL). Criteria for FISH anomalies were defined by use of histologically normal brain specimens as described previously (16,17). Simple gain required 10% or more of nuclei with three or more locus-specific probe signals. Loss of the q arm of chromosome 10 required the overall mean PTEN/CEP10 ratio to be less than 0.90. Amplification was applied only for EGFR hybridizations and required that the overall mean EGFR/CEP7 ratio must be 1.2 or more and that 10% or more of nuclei had more than three EGFR signals. Representative dual-probe (PTEN/CEP10) FISH images from two different tumors are shown in Fig. 1.

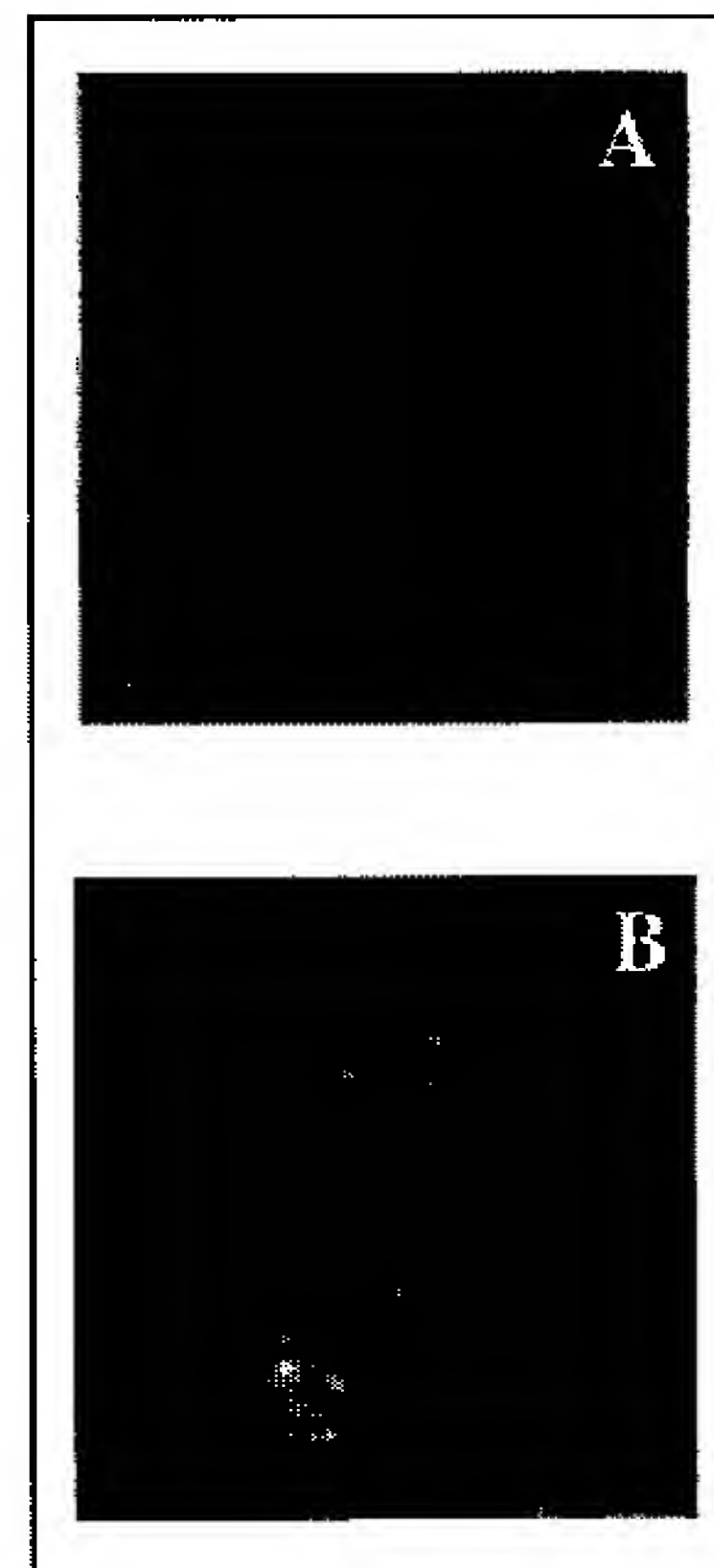
DNA for polymerase chain reaction (PCR)-based analysis was isolated from paraffin-embedded tumor sections by use of a microwave-based extraction method (18). Mutation analysis of all coding exons of PTEN and exons 5–8 of p53 was performed by directly sequencing PCR-amplified products (primer sequence available on request from the authors).

Tumors were screened for EGFR gene amplification by differential PCR by use of genomic primers for EGFR and genomic primers for the cystic fibrosis (CF) gene as described previously (19). EGFR/CF ratios of more than 2.0 were regarded as evidence for amplification (19). Tumors were screened for PTEN homozygous deletion by differential PCR by use of genomic primers for exon 5 of PTEN and genomic primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as described previously (20). PCRs and quantification of PCR products were performed under the conditions used for EGFR analysis, except that 2 and 0.5 pM of each PTEN and GAPDH primer pair, respectively, were used, and PTEN/GAPDH ratios of less than 0.3 were regarded as evidence for homozygous deletion (20).

Statistical Analysis

Frequency distributions and summary statistics were calculated for all clinical and tumor marker variables. For categorical variables, cross-tabulations were generated, and Wilcoxon tests (for ordered variables) and χ^2 tests (for nonordered

Fig. 1. Representative fluorescence *in situ* hybridization (FISH) images for two glioblastomas from the study set, one without EGFR gene amplification (A) and one with EGFR amplification (B). Representative FISH images of tumor nuclei dual hybridized with EGFR probe (red) and centromere 7 (CEN 7) probe (green) are shown.



variables) were used to compare their distributions. For continuous variables, Wilcoxon tests were used to investigate differences in the distributions between subsets of patients classified by categorical data (e.g., sex or p53 status). In addition, for several continuous variables, categorical variables were defined to create scientifically appropriate groups (e.g., age <40 years, 40–60 years, or >60 years). Tumors were considered to have EGFR amplification if either the FISH or PCR assays demonstrated this alteration. Likewise, tumors were considered to have PTEN alteration if sequencing or homozygous deletion assays exhibited an anomaly.

The Kaplan–Meier method (21) was used to estimate survival, defined as the time between study registration and a patient's death. In univariate survival analyses, two-sided log-rank tests (22) were used to assess the prognostic significance of age, sex, on-study performance score, extent of tumor resection, and abnormalities in EGFR, p53, and PTEN genes and chromosomes 7 and 10. Multivariate survival analyses to assess the prognostic significance of these same factors and to identify interactions among them were performed by use of classification and regression-tree (CART) models (23) in all 174 patients in the combined anaplastic astrocytoma/glioblastoma multiforme database. Subsequently, multivariate survival analyses by use of Cox proportional hazards models (24) were performed separately in the anaplastic astrocytoma and glioblastoma multiforme groups for the most promising factors on the basis of the CART model and univariate survival results. A bootstrap model selection procedure combining bootstrap methodology (25) with Cox model backward selection procedures (26) was used to validate the Cox model variable selection. Variables identified as statistically significant ($P < .05$) in 70% or more of the 500 bootstrap samples generated were considered to be valid prognostic indicators. All statistical tests were two-sided.

RESULTS

Patient Characteristics and Clinical Parameters

The study group consisted of 63 patients with anaplastic astrocytoma and 111 patients with glioblastoma multiforme. Table 1 compares the clinical characteristics of the anaplastic astrocy-

Table 1. Characteristics of patients with anaplastic astrocytoma (those evaluated for molecular genetic alterations [analyzed] versus those who lacked sufficient tissue for marker analyses [not analyzed])

Characteristic	Analyzed (n = 63)	Not analyzed (n = 62)	P*
Sex, No. (%)			.16†
Male	35 (56)	42 (68)	
Female	28 (44)	20 (32)	
Median age, y (range)	43 (14–79)	49.5 (23–77)	.10‡
On-study performance score, No. (%)			.24‡
0	14 (22)	22 (35)	
1	31 (49)	23 (37)	
2	11 (17)	13 (21)	
3	7 (11)	4 (6)	
Extent of resection, No. (%)			.30‡
Biopsy only	30 (48)	36 (58)	
Subtotal resection	24 (38)	20 (32)	
Gross total resection	8 (13)	6 (10)	
Protocol No., No. (%)			.014†
79-72-51	3 (5)	14 (23)	
85-72-51	33 (52)	26 (42)	
88-72-52	27 (43)	22 (35)	
Median survival, mo (range)	22.9 (0.1–147.8)	15.2 (0.3–143.5)	.23§
Median follow-up, mo (range)	104.7 (54.9–144.7)	95.3 (64.6–143.5)	.86‡
No. deceased (%)	51 (81)	53 (85)	.50†

*P values are from two-sided tests and were statistically significant when $P < .05$. Statistically significant values are in boldface type.

† χ^2 test (analyzed versus not analyzed groups).

‡Wilcoxon signed rank test.

§Log-rank test.

||Does not include deceased patients.

toma study group with those of the eligible patients with anaplastic astrocytoma who were not studied because of the lack of sufficient tissue blocks from their tumors. The majority of patients with anaplastic astrocytoma enrolled in the oldest trial (79-72-51) were not studied, reflecting the difficulty in obtaining tissue for these patients. The patients with anaplastic astrocytoma in the study set did not differ statistically significantly from the remaining patients with anaplastic astrocytoma with respect to distributions of age, sex, on-study performance score, extent of tumor resection, survival, follow-up time of living

Table 2. Incidence of genetic alterations in anaplastic astrocytomas versus glioblastomas*

Genetic alteration	Anaplastic astrocytomas	Glioblastomas	P†
EGFR amplification	11/63 (17)	46/111 (41)	.001
p53 point mutation	22/61 (36)	11/106 (10)	<.001
PTEN alteration	11/62 (18)	37/110 (34)‡	.033
Point mutation	11/62 (18)	32/110 (29)	.10
Homozygous deletion	0/62 (0)	11/110 (10)	.008
Gain of chromosome 7	25/62 (40)	43/111 (39)	.84
Loss of 10q§	4/55 (7)	48/107 (45)	<.001

*Number with alteration/number successfully analyzed (%).

† χ^2 or Fisher's exact test (anaplastic astrocytomas versus glioblastomas). Statistically significant values are in boldface type. All statistical tests were two-sided.

‡Six specimens had deletion by a differential polymerase chain reaction-based approach and point mutation.

§Of the specimens with the loss of the q arm of chromosome 10, apparent monosomy 10 was demonstrated in two (50%) anaplastic astrocytomas and in 42 (88%) glioblastomas.

patients, or the fraction deceased. Likewise, the patients with glioblastoma multiforme in the study set and the patients with glioblastoma multiforme who were not analyzed (data not shown) did not differ statistically significantly in any of these measurements.

Genetic Analysis

The incidence of genetic alterations in specimens of anaplastic astrocytoma and glioblastoma multiforme is shown in Table 2. EGFR gene amplification was statistically significantly ($P = .001$) less common in the anaplastic astrocytomas (17%) than in the glioblastomas (41%). There was no evidence of an association between the incidence of EGFR gene amplification and patient age (data not shown). In the tumors with EGFR gene amplification detected by FISH, the ratio of EGFR to centromere copy number ranged from 1.2 to 10.0 (mean = 4.0) in the anaplastic astrocytomas and from 1.5 to 11.1 (mean = 6.5) in the glioblastomas. Differential PCR and FISH analyses detected 39 and 49 specimens with EGFR amplification, respectively, and both methods detected EGFR amplification in 35 specimens. The concordance and correlation coefficient between FISH and

Fig. 2. Association of EGFR gene amplification, p53 point mutation, and PTEN alteration in 61 anaplastic astrocytomas (A) and in 105 glioblastomas (B). An inverse association was identified between EGFR gene amplification and p53 point mutation in glioblastomas ($P = .023$). No specimen had all three alterations. All statistical tests were two-sided.

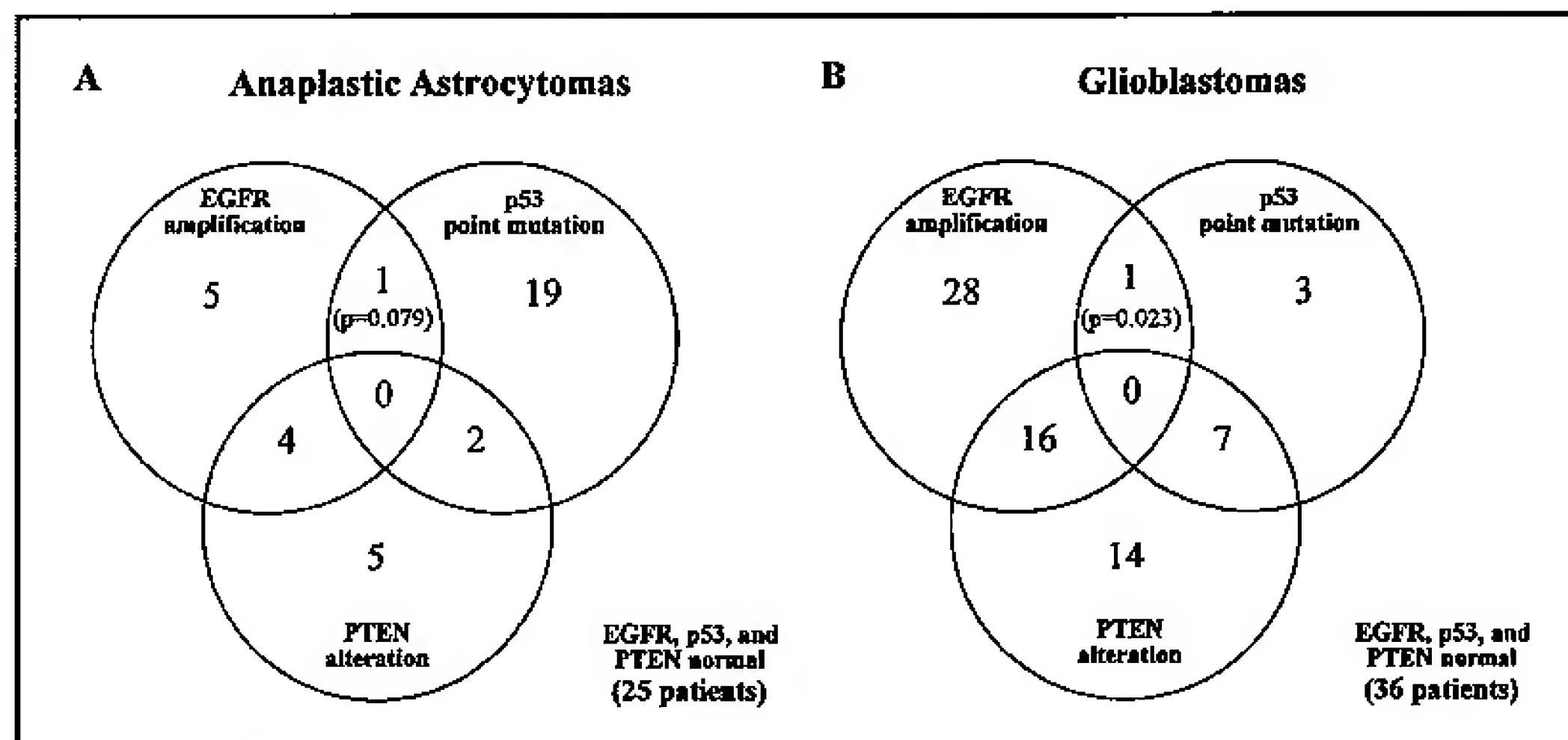


Table 3. Univariate associations of clinical and genetic factors with survival

	Anaplastic astrocytomas				Glioblastomas				Anaplastic astrocytoma and glioblastoma multiforme	
	No. of patients	No. of deaths (%)	Median survival, mos	<i>P</i> *	No. of patients	No. of deaths (%)	Median survival, mos	<i>P</i> *	Median survival, mos	<i>P</i> *
Age, y				<.001				<.001		<.001
<40	25	16 (64)	65.5		9	9 (100)	12.3		36.6	
40–60	23	20 (87)	24.2		58	56 (97)	14.3		15.8	
>60	15	15 (100)	4.4		44	44 (100)	8.4		8.0	
Sex, No.				.21				.70		.13
Male	35	30 (86)	19.2		75	73 (97)	11.7		12.2	
Female	28	21 (75)	26.4		36	36 (100)	11.7		14.3	
Performance score†				.030				.017		.003
0	14	10 (71)	32.3		29	28 (97)	15.0		16.2	
1	31	24 (77)	36.4		54	53 (98)	10.9		12.6	
2–3	18	17 (94)	6.3		28	28 (100)	8.6		8.6	
Extent of resection				.81				.31		.94
Biopsy	30	24 (80)	19.2		32	32 (100)	10.2		12.8	
Subtotal	24	20 (83)	38.7		55	54 (98)	11.5		12.3	
Gross total	8	6 (75)	18.5		24	23 (96)	11.7		13.0	
EGFR				.24				.58		.089
Amplification	11	10 (91)	22.1		46	46 (100)	12.6		12.7	
No amplification	52	41 (79)	22.9		65	63 (97)	10.2		12.5	
p53				.012				.33		<.001
Point mutation	22	16 (73)	59.0		11	9 (82)	11.7		38.7	
Wild-type	39	33 (85)	16.0		95	95 (100)	11.5		11.6	
PTEN				.002				.74		.001
Alteration	11	11 (100)	4.4		37	35 (95)	11.7		10.4	
No alternative	51	39 (76)	34.4		73	73 (100)	11.2		14.7	
Chromosome 7				.60				.76		.98
Gain	25	19 (76)	16.0		43	43 (100)	11.5		12.1	
No gain	37	31 (84)	26.4		68	66 (97)	11.7		13.6	
Chromosome 10 q-arm				.97				.65		.030
Hemizygous loss	4	3 (75)	22.1		48	48 (100)	12.2		12.4	
No hemizygous loss	51	43 (84)	26.4		59	57 (97)	10.5		14.6	

P* values are from two-sided tests and were statistically significant when $<.05$. Statistically significant values are in **boldface type.

†Baseline performance score.

differential PCR for the detection of EGFR amplification were 89% and .73, respectively.

p53 point mutations were statistically significantly ($P<.001$) more common in the anaplastic astrocytomas (36%) than in the glioblastomas (10%). Within the glioblastoma multiforme study group, younger patients were more likely to have p53 point mutation ($P = .007$); a similar trend was observed within the anaplastic astrocytoma study group but was not statistically significant ($P = .14$; data not shown). All mutations were missense mutations except for one nonsense mutation in an anaplastic astrocytoma and two in-frame deletions or insertions in two glioblastomas. The distribution of missense mutations was similar between the anaplastic astrocytoma and glioblastoma multiforme study groups (data not shown) and was consistent with previous reports (27).

PTEN alteration was statistically significantly ($P = .033$) less frequent in anaplastic astrocytomas (18%) than in the glioblastomas (34%). PTEN alteration was statistically significantly more common among older patients with anaplastic astrocytoma ($P = .014$) and statistically significantly more common among younger patients with glioblastoma multiforme ($P = .008$; data not shown). No apparent clustering of missense mutations was identified, and nonsense and frameshift mutations were primarily confined to exons 7 and 8 (data not shown). All homozygous

deletions identified were in specimens of glioblastoma multiforme (10%; $P = .008$).

Gain of chromosome 7 was identified in approximately 40% of both anaplastic astrocytoma and glioblastoma multiforme study groups ($P = .84$). There was no evidence of an association between the incidence of chromosome 7 gain and patient age (data not shown).

Hemizygous deletion of the q arm of chromosome 10 was statistically significantly ($P<.001$) less common in anaplastic astrocytomas (7%) than in glioblastomas (45%). In the tumors with loss of 10q, apparent loss of an entire copy of chromosome 10 was identified in 50% of anaplastic astrocytomas and in 88% of glioblastomas. There was no evidence of an association between the incidence of hemizygous deletion of 10q and patient age (data not shown).

Fig. 2 presents Venn diagrams of EGFR gene amplification, p53 mutation, and PTEN alteration for the 61 anaplastic astrocytoma specimens and 105 glioblastoma multiforme specimens for which all three parameters were assessed successfully. No specimens had all three alterations. EGFR gene amplification and p53 point mutation rarely occurred together; both alterations were found in only one anaplastic astrocytoma (2%) and in one glioblastoma multiforme (1%). The two alterations that occurred together most frequently were EGFR gene amplification and

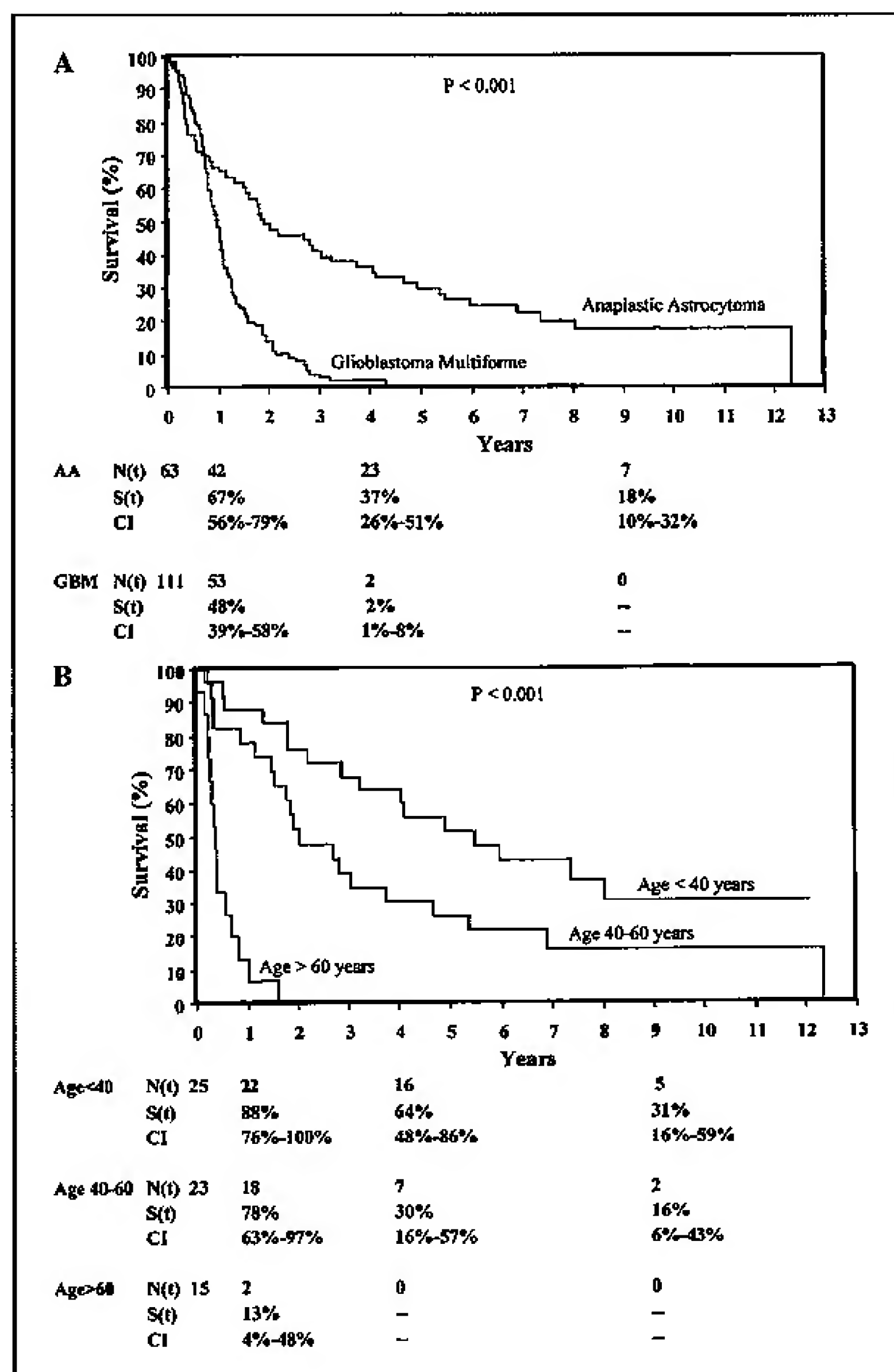


Fig. 3. Survival curves according to histologic tumor grade (A) and for the anaplastic astrocytoma patient subsets defined by the following: patient age (B), EGFR gene amplification (C), and status of PTEN and p53 (D). All statistical tests were two-sided. N(t) and S(t) indicate, respectively, the number of patients at risk and the Kaplan-Meier estimate of survival at time *t*. CI = 95% confidence interval for the Kaplan-Meier survival estimate.

PTEN alteration, which were found together in four anaplastic astrocytomas (6%) and in 16 glioblastomas (15%). Among the 62 anaplastic astrocytomas and 111 glioblastomas whose specimens could be assessed for both EGFR gene amplification and chromosome 7 gain (data not shown), no association was detected between these markers in the anaplastic astrocytomas ($P = .74$), but the markers were positively associated in the glioblastomas ($P = .018$).

Univariate Analysis of Survival

Among the patients with anaplastic astrocytoma, survival was found to be statistically significantly associated with age (log-rank $P < .001$), on-study performance score ($P = .030$), p53 status ($P = .012$), and PTEN status ($P = .002$) by univariate analysis (Table 3). p53 mutation was associated with longer survival, and PTEN alteration, old age, and poor performance score were associated with shorter survival. Patients with EGFR gene amplification exhibited a trend toward a shorter survival, although the difference was not statistically significant ($P = .24$).

Fig. 3 shows the Kaplan-Meier survival curves for patients with anaplastic astrocytoma versus patients with glioblastoma multiforme and for the anaplastic astrocytoma subsets defined by age, EGFR gene amplification, PTEN alteration, and p53 mutation.

In the patients with glioblastoma multiforme, univariate analyses of the nine variables in Table 3 found that survival was statistically significantly associated with age ($P < .001$) and on-study performance score ($P = .017$). There was no evidence of an association between survival and any of the tumor markers.

Multivariate Analysis of Survival

We performed a series of multivariate analyses that demonstrated that PTEN and EGFR alterations are independent predictors of survival in this series of glioma patients. CART modeling was performed in the total group of 174 anaplastic astrocytoma and glioblastoma multiforme specimens to identify subsets of patients with distinctly different survival distributions on the basis of histologic grade and the nine independent variables listed in Table 3. CART procedures generate a tree con-

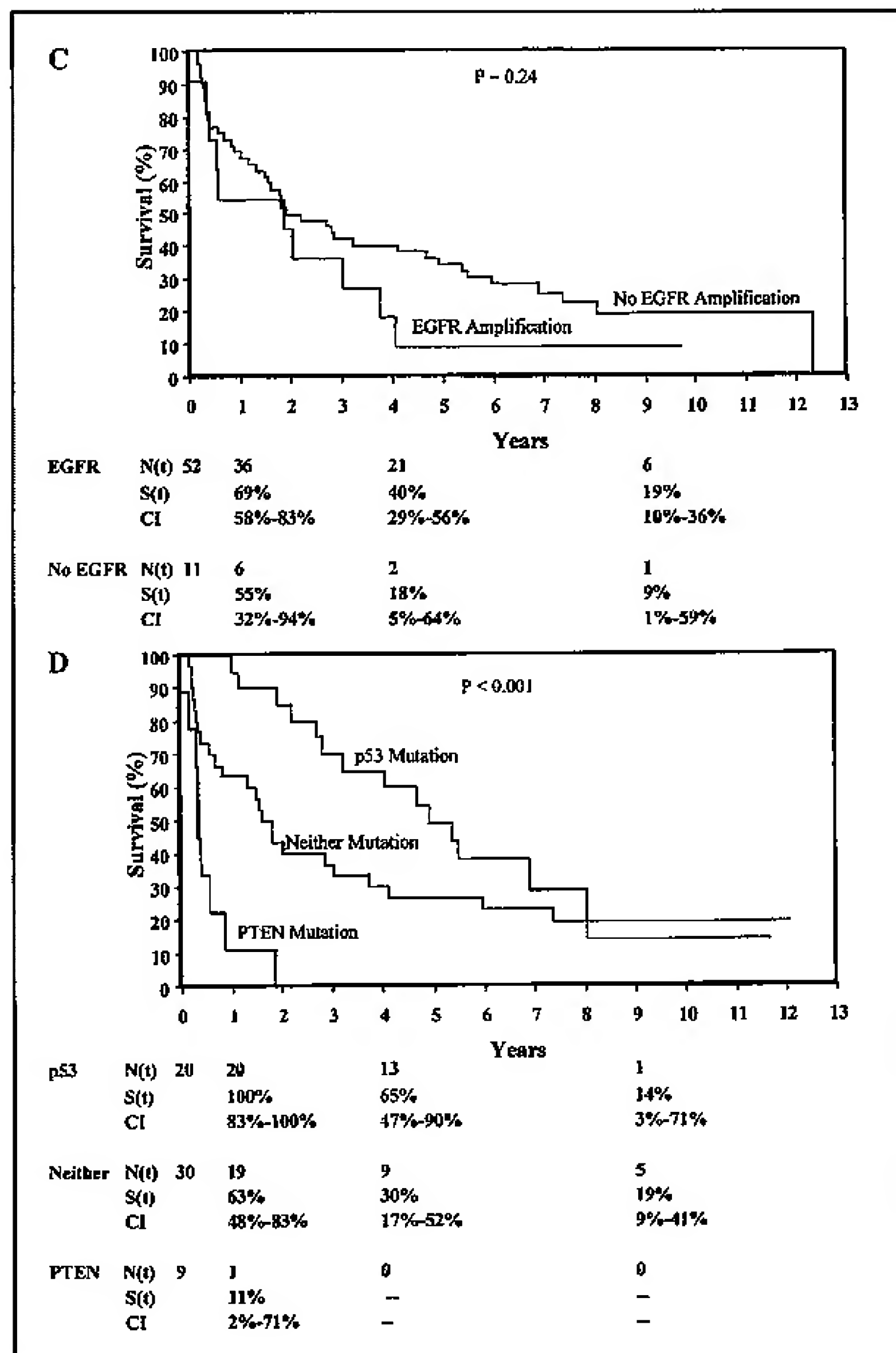


Fig. 3. (Continued).

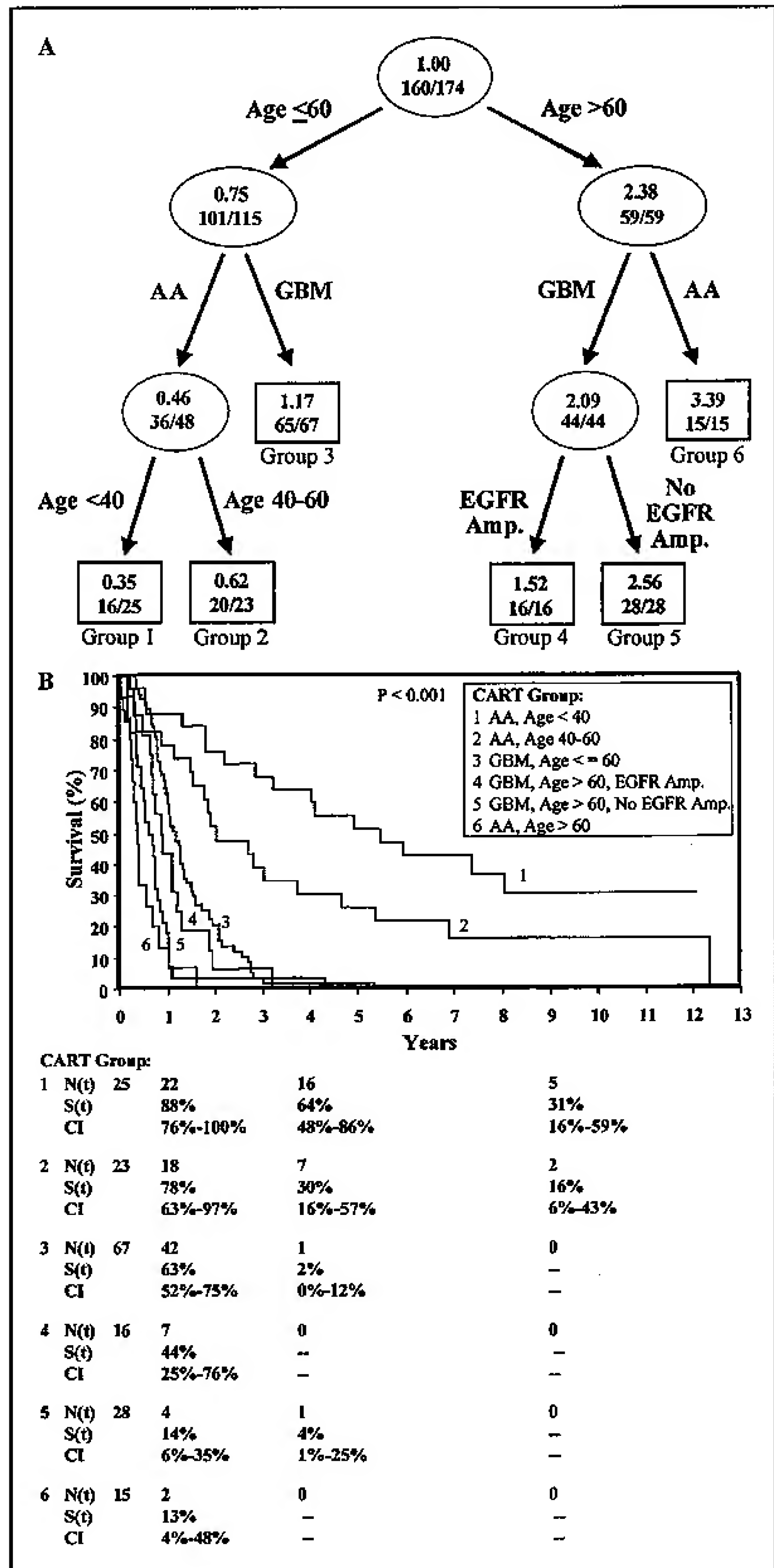
sisting of lymph nodes (subsets of patients) by successively splitting each lymph node into two lymph nodes corresponding to the various ways that the values of each independent baseline variable can be split into two groups. The variable that splits a given lymph node into two subsets with the greatest difference in survival is chosen as the optimum split for that lymph node. This technique can identify interactions between independent baseline variables.

For the total group of 174 patients, CART procedures identified six groups of patients with distinctly different survival distributions (Fig. 4, A). Fig. 4, B, shows the Kaplan-Meier survival curves for these six groups. Those with the best survival were the 25 patients with anaplastic astrocytoma who were younger than 40 years old, and those with the worst survival were the 15 patients with anaplastic astrocytoma who were older than 60 years. Notably, the CART model shows that EGFR amplification is associated with a better survival in the 44 patients with glioblastoma multiforme who were older than 60 years. However, the CART modeling process also revealed that

EGFR amplification is associated with worse survival in patients who were 60 years old or younger (data not shown), although this association was not strong enough for inclusion in the final CART model. PTEN and p53 alterations were also consistently shown to be moderately associated with poorer survival and with better survival, respectively.

Backwards Cox proportional hazards modeling procedures were then applied with independent variables that were chosen from the results of the univariate analyses and CART models described above: Given the relatively small number of anaplastic astrocytomas, three variables (sex, chromosome 7 gain, and chromosome 10q loss) were omitted because of the lack of evidence of association with survival and (for 10q loss) a higher percentage of missing data. Therefore, five clinical variables (age <40 years, age >60 years, on-study performance score, biopsy, and gross total resection) and three tumor markers (status of EGFR, p53, and PTEN) were used. The best model for a given patient group was obtained by starting with the Cox model containing all eight variables and successively eliminating the

Fig. 4. A) Classification and regression-tree (CART) survival analysis to identify which of 10 variables (age, sex, on-study performance score, extent of tumor resection, histologic tumor grade, EGFR gene amplification, PTEN alteration, p53 mutation, chromosome 7 gain, and loss of the q arm chromosome 10) were most strongly associated with survival in the combined population of 63 anaplastic astrocytomas (AAs) and 111 glioblastomas (GBMs). Ovals and square boxes indicate, respectively, intermediate and terminal subsets of patients defined by the sequential splitting process. The lower number within each oval or square indicates the number of deaths and patients in that subset. The upper number is the normalized hazard rate for the subset obtained by dividing the constant hazard rate for the subset by the constant hazard rate for the total group ($n = 174$), calculated by use of rescaled survival times. (CART rescales the total set of survival times to fit an exponential model, i.e., so that the Kaplan-Meier survival curve of all 174 patients is a straight line when plotted on the logarithmic scale.) For each square box, the group number refers to the corresponding survival curve in panel B. The variable used for each split is noted. Amp = amplification. B) Survival curves for the six groups identified by CART survival analysis of the combined population of AA and GBM patients. The groups are patients with 1) AA who were younger than 40 years, 2) AA who were 40–60 years old, 3) GBM who were 60 years old or younger, 4) GBM who were older than 60 years with EGFR amplification, 5) GBM who were older than 60 years without EGFR amplification, and 6) AA who were older than 60 years. All statistical tests were two-sided. $N(t)$ and $S(t)$ indicate, respectively, the number of patients at risk and the Kaplan-Meier estimate of survival at time t . CI = 95% confidence interval for the Kaplan-Meier estimate.



least statistically significant variables until only statistically significant variables ($P < .05$) were left. Table 4 summarizes the Cox modeling process and results in each patient group.

Table 4 shows that, in the total group of patients with anaplastic astrocytoma and glioblastoma multiforme, p53 and

PTEN mutations were statistically significant prognostic factors after adjustment for the effects of on-study performance score and age. The fact that EGFR was not statistically significant in the total-group Cox model is not surprising, given the interaction between EGFR and age found by CART. However, only

Table 4. Cox backward elimination survival models, by patient group

Independent variable	Best Cox models* for indicated cohorts of patients†								
	All tumors studied (n = 165)			Anaplastic astrocytomas (n = 60)			Glioblastomas (n = 105)		
	Hazard ratio			Hazard ratio			Hazard ratio		
	P	Point estimate	95% CI	P	Point estimate	95% CI	P	Point estimate	95% CI
Young, age <40 y	.003	0.48	0.29 to 0.78	.042	0.48	0.24 to 0.98	X		
Old, age ≥60 y	.001	2.54	1.74 to 3.69	<.001	7.52	3.02 to 18.73	.008	1.75	1.16 to 2.65
Biopsy, yes/no	X			X			X		
Gross total resection, yes/no	X			X			X		
First performance score, 0–4	.008	1.30	1.07 to 1.57	X			.005	1.42	1.11 to 1.82
Anaplastic astrocytoma, yes/no	X			ND‡			ND		
EGFR amplification, yes/no	X			X			X		
p53 mutation, yes/no	.011	0.55	0.35 to 0.87	X			X		
PTEN mutation, yes/no	.001	2.11	1.46 to 3.06	<.001	4.34	1.82 to 10.34	X		

*The best model for a given patient cohort was obtained by successively eliminating the least statistically significant variables from the full Cox model containing all eight (or nine) independent variables until only statistically significant ($P < .05$) variables were left.

†For variables statistically significantly associated with survival in the best Cox model for a given patient cohort, the corresponding P value, hazard ratio point estimate, and 95% confidence interval (CI) are shown. Factors with hazard ratios of less than 1.0 (or >1.0) are associated with better (or worse) survival. The P value of variables not statistically significantly associated with survival in the best Cox model are indicated by an X.

‡ND = variable was not included in the full Cox model for the patient cohort.

PTEN was statistically significantly prognostic after adjustment for age in the patients with anaplastic astrocytoma (hazard ratio = 4.34; 95% confidence interval [CI] = 1.82 to 10.34) but not in the patients with glioblastoma multiforme, and p53 was not statistically significantly prognostic in either group. Bootstrapping procedures applied to the Cox modeling process confirmed the prognostic significance of age 60 years or older and PTEN status in the patients with anaplastic astrocytoma (data not shown).

In the patients with glioblastoma multiforme, the best model incorporated two parameters (i.e., age ≥60 years and performance score). Both were validated by bootstrapping procedures.

DISCUSSION

The 174 specimens of high-grade astrocytomas studied were from all 63 patients with biopsy-proven anaplastic astrocytoma and sufficient tissue for these marker studies who were enrolled in one of the three consecutive NCCTG phase-III trials for newly diagnosed high-grade gliomas (10–12) and all 111 Mayo patients with biopsy-proven glioblastoma multiforme and sufficient tissue for marker ascertainment who were enrolled in the same three NCCTG trials or six concurrent MCCC trials in newly diagnosed high-grade glioma or an NCCTG-led intergroup trial in newly diagnosed glioblastoma multiforme. With regard to clinical characteristics, the patients with available tumor tissue did not differ substantially from those without adequate tissue for analysis. In addition, this series of patients is broadly typical of other series of patients with high-grade astrocytoma with regard to clinical features and incidences of genetic alterations (1,28–31).

Of the molecular markers analyzed in this study, alteration of the PTEN tumor suppressor gene showed the strongest association with survival in patients with anaplastic astrocytoma. Patients with anaplastic astrocytoma who had a PTEN alteration had statistically significantly worse survival than patients with-

out PTEN alteration, both in univariate analyses and in multivariate model analyses after adjustment for key clinical variables, i.e., age, on-study performance score, and extent of resection. Although previous studies have suggested an association between PTEN and survival in patients with astrocytic glioma, to our knowledge, this is the first report of an association between PTEN gene alteration and survival among adult patients with anaplastic astrocytoma. Lin et al. (32) noted an association between hemizygous deletion of the region surrounding the PTEN locus and shorter survival among patients with high-grade glioma. Sano et al. (33) noted a statistically significantly better prognosis for patients with glioblastoma multiforme whose tumors expressed high levels of PTEN messenger RNA. Furthermore, Raffel et al. (34) reported an association between PTEN mutation and survival among pediatric patients with high-grade astrocytoma, after adjustment for the effects of patient age and tumor grade. Although two studies (35,36) identified no association between survival and point mutation of PTEN among patients with high-grade astrocytic glioma, neither of these studies examined a large number of patients with anaplastic astrocytoma.

Mutation of PTEN has been implicated in the malignant progression of astrocytic gliomas, because these alterations are observed most frequently in glioblastoma multiforme, less commonly in anaplastic astrocytoma, and only rarely in low-grade astrocytoma (36). PTEN mutation among the patients with anaplastic astrocytoma in this series conferred a median survival that was similar to that of the patient population with glioblastoma multiforme. Consequently, PTEN analysis of anaplastic astrocytomas may distinguish those tumors that have acquired the genetic features of glioblastoma multiforme but have yet to show their characteristic histologic aberrations.

Assessment of PTEN may assist in appropriately classifying patients with anaplastic astrocytoma for therapeutic trials. Specifically, analysis of therapies that may benefit patients with anaplastic astrocytoma could be adversely affected by inclusion of patients whose disease was classified histologically as ana-

plastic astrocytoma but exhibiting PTEN mutation. Thus, we suggest that assessment of PTEN status should be incorporated into future clinical trials in patients with anaplastic astrocytoma to verify its prognostic significance and to identify any difference in therapeutic benefit in patients with and without PTEN alteration.

In addition to patient age and PTEN alteration, univariate analyses of the anaplastic astrocytoma study group identified mutation of p53 as a statistically significant predictor of prolonged survival. This association is consistent with previous observations that mutation of p53 is more frequent among younger patients with astrocytic glioma (37). However, after adjustment for the effect of patient age, p53 mutation was not associated with survival in the anaplastic astrocytoma study group, in accordance with the majority of previous reports (1).

Gain of chromosome 7 is a frequent event in both low- and high-grade astrocytic glioma (28,29), suggesting that this alteration is an early event in astrocytoma development. Huhn et al. (29) reported an association between gain of chromosome 7 and radiation resistance in glioblastoma multiforme, suggesting that this aberration may predict poor prognosis. However, gain of chromosome 7 was not associated with survival in either the anaplastic astrocytoma or glioblastoma multiforme patient groups in this series.

Deletion of the q arm of chromosome 10 has been reported in approximately 80% of high-grade astrocytomas (9), and several candidate tumor suppressor genes have been identified from this chromosome arm, including PTEN (38–40). However, deletion of 10q was not predictive of survival in patients with anaplastic astrocytoma or glioblastoma multiforme.

In the glioblastoma multiforme study group, the statistically significant predictors of overall survival identified by both univariate and multivariate analyses were patient age and on-study performance score. None of the genetic markers analyzed in this study was identified as a statistically significant predictor of survival among patients with glioblastoma multiforme. Indeed, other investigators (5,7) have obtained similar results. Inability to distinguish differences in survival among patients with glioblastoma multiforme based on genetic markers associated with malignant progression suggests that there may be multiple genetic pathways of glioblastoma multiforme development that converge on a similar phenotype with an equally poor prognosis.

As an alternative means of assessing prognostic indicators in our study group, clinical and molecular data from all 174 specimens (63 anaplastic astrocytomas and 111 glioblastomas) were subjected to CART-survival modeling procedures. This analysis generates a regression tree consisting of lymph nodes by successively splitting each lymph node based on the variable that distinguishes the greatest difference in survival. Initial splitting was based on patient age. Subsequent splitting in the older patients (age >60 years) indicated particularly poor survival for older patients with anaplastic astrocytoma and longer survival in older patients with glioblastoma multiforme who also had EGFR gene amplification. However, in the younger patients (age ≤60 years), the splitting process consistently indicated shorter survival in younger patients with EGFR amplification, although this association was not strong enough for inclusion in the final CART model. These observations suggest that EGFR gene amplification may have prognostic utility and that the biologic significance of EGFR gene amplification in astrocytic gliomas may be related to patient age, as reported by Simmons et al. (41). For

example, when EGFR is amplified in gliomas, it is often also mutated (42). It is possible that different mutations (e.g., 3' deletion, 5' deletion, or various point mutations) occur with different incidence in various glioma malignancy grades or in tumors from patients of various ages.

We observed a paucity of specimens with both EGFR amplification and p53 mutation, which lends support to the hypothesis that p53 mutation and EGFR gene amplification are markers for two pathways of astrocytic glioma development or two alternative alterations in the same pathway (9,37). It is interesting to note that the incidence of p53 mutation was statistically significantly less in the glioblastoma multiforme specimens than in the anaplastic astrocytoma specimens in this study. Similar results have been reported previously (37) and suggest that the NCCTG cohort of patients with glioblastoma multiforme includes a large proportion of "primary" glioblastomas.

Perry et al. (43) recently analyzed multiple clinicopathologic variables in the same anaplastic astrocytoma patient group examined in this study and observed that foci of necrosis and/or endothelial hyperplasia were identified in five of the patients with anaplastic astrocytoma when tissue blocks were recut to obtain deeper sections. When we reanalyzed the survival of the remaining 58 patients with pure anaplastic astrocytoma, the median survival time increased from 23 to 29 months, but none of the conclusions regarding the clinical and molecular genetic variables changed (data not shown). This finding emphasizes the histologic grading difficulties introduced by the heterogeneity of these tumors and exemplifies the need for more objective tumor markers. Furthermore, because 48% of our patients with anaplastic astrocytoma were diagnosed on biopsy only, the risks of undersampling are substantial. However, because this reflects the practices of both community and academic neurosurgical practices, it should be regarded as a "realistic flaw" associated with the evaluation of astrocytic neoplasms. Notably, just one of the five anaplastic astrocytoma specimens with focal necrosis or endothelial proliferation had a PTEN alteration.

In summary, we have assessed the prognostic value of several genetic alterations in patients with high-grade astrocytic glioma. Key attributes of our patient population include mature follow-up, protocolized prospective follow-up, and relatively large sample sizes. The most clinically significant findings of this study are that patients with anaplastic astrocytoma who had an alteration of the PTEN tumor suppressor gene had a statistically significantly shorter survival than patients with anaplastic astrocytoma without PTEN mutation and that this association was maintained after adjustment for the effects of key clinical variables, including patient age, on-study performance score, and extent of tumor resection. In addition, the presence of EGFR amplification independently predicts a longer survival in older patients with glioblastoma multiforme. The substantial variation in survival among the patients with anaplastic astrocytoma suggests that combining histologic and genotypic assessment could potentially improve existing strategies for patient stratification and management.

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NOTES

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